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⑳ Glycolytic promoters for regulated protein expression: protease inhibitor.

㉑ Promoters associated with expression of specific enzymes in the glycolytic pathway are used for expression of alien DNA, particularly yeast promoters known to provide high enzyme levels of enzymes in the glycolytic pathway are employed for expressing a mammalian protein, such as alpha-1-antitrypsin. The promoters include promoters involved in expression of pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase, phosphoglycerate mutase, hexokinase 1, hexokinase 2, glucokinase, phosphofructose kinase, and aldolase, as well as the glycolytic regulation gene. Particularly, the glycolytic regulation gene can be used in conjunction with promoters in the glycolytic pathway for regulated production of desired proteins.

**EP 0 103 409 A2**

GLYCOLYTIC PROMOTERS FOR REGULATED PROTEIN  
EXPRESSION: PROTEASE INHIBITOR

The ability to obtain expression of foreign, i.e., exogenous, DNA in unicellular microorganisms provided  
5 the opportunity to conveniently prepare long polypeptide chains of interest. Almost immediately, varied polypeptides, such as the small hormone somatostatin and more sophisticated polypeptides, such as insulin, interferons, thymosin and a variety of vaccines having capsid  
10 proteins, were prepared and reported in the literature. For the most part, the initial work was performed in the bacterium E. coli which had been the subject of intensive study because scientists were familiar with many aspects of its genetic structure and properties.  
15 Initial attention was therefore directed to producing foreign proteins in E. coli. Once the ability to employ E. coli as a host was established, the limitations and disadvantages of employing E. coli encouraged the use of other hosts.

20 One host which appeared to be particularly attractive because it lacked many of the shortcomings of E. coli was yeast. However, yeast is a eukaryote and, therefore, has a more sophisticated genetic system. Furthermore, less is known about the yeast genome than is known  
25 about E. coli. In order to use yeast as a host for the production of proteins foreign to yeast, a number of discoveries are required, and new materials must be made available.

Initially, a replication system was required which provided stability in yeast, either as an extrachromosomal element or by integration into the yeast chromosome. In addition, the regulatory functions concerned with transcription and expression had to be developed in order to allow for expression of the desired protein. There was also the uncertainty whether foreign DNA sequences would be transcribed and translated and, if expressed, whether the resulting polypeptides would survive in the yeast cell. Also remaining to be determined was the effect of the foreign proteins on the viability of the yeast cell, such as the effect of recombinant DNA (RDNA) on mitosis, sporulation and vegetative growth.

There have, therefore, been substantial efforts to develop novel RDNA systems in yeast, which will allow for regulated expression of a protein of interest, as well as highly efficient production of such proteins.

Hitzeman et al., J. Biol. Chem., 255:12073-12080 (1980) describe a plasmid having a yeast 3-phosphoglycerate kinase (PGK) gene and accompanying regulatory signals capable of expression in yeast. Other references of interest include Clifton, et al., Genetics, 88:1-11 (1978); Clark and Carbon, Cell, 9:91-99 (1976); Thomson, Gene, 1:347-356 (1977); Holland and Holland, J. Biol. Chem., 254:5466-5474 (1979); Holland and Holland, ibid. 254:9830-9845 (1979); Nasmyth and Reed, Proc. Nat. Acad. Sci., 77:2119-2123 (1980); Broach, et al., Gene, 8:121-133 (1979); and Williamson, et al., Nature, 283:214-216 (1980).

In the accompanying FIGURES:

FIGS. 1A and 1B are cDNA sequences of two forms of genes coding human alpha-1-antitrypsin.

FIG. 2 illustrates the restriction maps of plasmids CTEA32 and CAT1.

FIG. 3 is a diagram of the electrophoresis chromatogram showing purified alpha-1-antitrypsin produced according to the present invention.

FIG. 4 illustrates the restriction map of plasmid C1/1.

FIG. 5 illustrates the DNA sequence of the multiple restriction site of pUC13.

FIG. 6 illustrates the restriction map of plasmid pUC $\alpha$ 1 containing the DNA sequence from FIG. 1.

FIG. 7 is the restriction map of plasmid HAT4.

Novel yeast promoters are provided which control the transcription of genes in the glycolytic pathway and which find use in the regulated production of proteins foreign to the yeast. Promoters of particular interest include the promoters for triose phosphate isomerase, pyruvate kinase, phosphoglucose isomerase, phosphoglycerate mutase, hexokinase 1, hexokinase 2, glucokinase, phosphofructo kinase, and aldolase, as well as the glycolytic regulatory gene. The protease inhibitor, mammalian alpha-1-antitrypsin, is expressed using the promoter for triose phosphate isomerase.

Methods and compositions are provided for regulated efficient expression of alien or foreign DNA in a yeast host. (Alien or foreign DNA is DNA not naturally occurring in the wild type particularly from a different species and which does not normally exchange genetic information with the host.) Novel promoters are employed which are involved in the glycolytic pathway and provide for high levels of protein production, so that a

substantial proportion of the total protein produced by the yeast cells can be dedicated to the protein of interest. In addition, regulatory mechanisms associated with regulation of production of the glycolytic enzymes  
5 are achieved, so that production of the desired products may be modulated. Furthermore, viable cells can be maintained to enhance the efficiency and amount of expression.

The promoters of interest are particularly those pro-  
10 moters involved with expression of triose phosphate isomerase, pyruvate kinase, phosphoglucose isomerase, phosphoglycerate mutase, hexokinase 1, hexokinase 2, glucokinase, phosphofructo kinase, and aldolase, which are controlled by the glycolytic regulation gene GCR1.  
15 The genes of the glycolytic pathway include hexokinase 1 and 2 (HXK1,2); phosphoglucose isomerase (PGI), triose phosphate isomerase (TPI); phosphoglycerate kinase (PGK), phosphoglycerate mutase (GPM), pyruvate kinase (PYK), phosphofructo kinase (PFK), enolase (ENO); fructose  
20 1,6-diphosphate aldolase (FDA); glyceraldehyde 3-phosphate dehydrogenase (PGK); and glycolysis regulation protein (GCR).

The promoters may be obtained by employing a gene bank having large fragments of yeast DNA. By introducing the  
25 fragments into appropriate vectors, particularly shuttle vectors having replicons for prokaryotes and yeast, one can readily amplify and clone the yeast DNA in a bacterium and then introduce the yeast DNA into mutant yeast cells for complementation. In this manner, yeast  
30 fragments can be identified which complement auxotrophic lesions or mutations in a yeast host.

Of particular interest, is where the host is auxotrophic in both the glycolytic pathway step of interest and a separate biochemical pathway, which is complemented by a

marker in the vector. Once having established a DNA segment having the desired gene, one may reclone by various techniques to shorten the DNA segment and provide for a segment which is primarily the gene of interest in conjunction with its regulatory signals for transcription and expression.

In order to retain the promoter, it is essential that the initiator methionine be determined and this codon be used for developing the strategy for introducing the 10 alien DNA downstream from the promoter. Various techniques can be employed for providing a site for introduction of the alien DNA so as to be under the regulatory control of the promoter in the glycolytic pathway.

Where a restriction site is conveniently adjacent to the 15 initiator methionine codon, the glycolytic gene may be cleaved at that site and the DNA chewed back with Bal31 for varying periods of time, so as to chew into or past the initiator methionine codon or retain the initiator methionine codon.

20 Where there is no convenient restriction site, other splicing techniques such as primer repair may be employed. Also, by employing in vitro mutagenesis, one can introduce a restriction site adjacent the initiator methionine, which encodes for the initial amino acids of 25 the desired protein. In each instance, a linearized DNA segment is obtained having the intact promoter for the glycolytic product and normally includes other DNA sequences, such as an intact replicon, one or more markers, and the like.

30 Exemplary of the above procedure is the development of a vector having the promoter for the TPI1 gene. An exemplary vector CV13 having the replicons or replica-

tion systems from pBR322 and 2 $\mu$ -plasmid of yeast, as well as the LEU2 gene was employed for insertion of a yeast fragment which was shown to have the TPI1 gene. This was achieved by employing double selection with a mutant yeast which was leu<sup>-</sup>, tpi<sup>-</sup>. The TPI1 gene was found to have a unique KpnI site. The vector was cleaved at the KpnI site and then treated with the double stranded exonuclease Bal31 for varying times to chew back the DNA to about the f-met codon. Linkers  
10 were then inserted providing desired restriction sites. Alien DNA could then be inserted providing a sequence having a f-met codon in the appropriate position for initiation. Alternatively, the foreign DNA can be expressed using the f-met codon of the TPI1 gene.

15 Similar procedures can be performed with the other subject glycolytic genes in order to provide the promoters associated with those genes. The PYK sequence has a convenient XbaI site for restriction, where the few additional bases may be removed, if required, using  
20 Bal31 for a short period of time to chew to or through the methionine codon. Of particular interest is the use of the GCR promoter to control the expression of the other genes involved in the glycolytic pathway. By employing the GCR gene, in conjunction with other  
25 glycolytic promoters regulating expression of alien DNA, one can turn on and off the other promoters, so as to regulate the expression of the alien DNA. Thus, one can allow vegetative growth to proceed until a desired cell density is achieved, before permitting production of the  
30 desired polypeptide.

By employing appropriate auxotrophs, one can further regulate the expression of the polypeptides of interest in choosing the appropriate nutrient medium. Where the chosen promoter is repressed by the particular nutrient  
35 because of a metabolic block, a change in the nature of

the nutrient can induce expression. Furthermore, the activity of a number of promoters in the glycolytic pathway can be affected by the repression or activation of expression by the GCR gene or other regulatory controls. Also, the GCR regulatory signals can be used to titrate the polypeptide functioning as the regulator for expression of GCR. By having vectors whose copy number can be controlled, one can vary the activity of the wild type GCR gene.

10 In order to obtain expression, an extrachromosomal element construct will be prepared having a number of sequences defining different functions. One function is the replication system, which forms part of a vector. Another function is a promoter by itself or in conjunction with the alien DNA. Other functions include initiators and terminators of expression. Also, there will be selectable markers.

In developing an appropriate vector, while not necessary, it will be common to have both a replication system for yeast and a replication system for a prokaryote (a shuttle vector). The replication system for yeast may be one which provides for stable maintenance of an extrachromosomal element or one which provides a sufficient lifetime for the DNA in the host, that there is an acceptable probability of integration of the DNA into the host. Integration can be greatly aided by providing for a sequence homologous to the host DNA, so as to provide for recombination. Generally, the homologous sequence will be at least about 800bp usually not more than about 2000bp. Therefore, either integration or an autonomous replication system, such as the use of the ARS1 gene, may be employed to provide for the maintenance of the alien DNA in the yeast host. The replication system which is chosen should provide for a reasonable copy number usually greater than 1, pref-



erably greater than 5. A wide variety of replication systems are available on a wide variety of prokaryotic vectors, such as pBR322, pACYC184, pSC101, pMB9, etc. Alternatively, one or more copies of the DNA construct 5 can be integrated into the host chromosome. The replication systems may also be conditionally regulated, usually being temperature sensitive so that replication can be turned on and off by varying the temperature.

In addition to the replication system, there will also 10 be one or more selectable markers, there usually being at least one marker in addition to the alien DNA, which may serve as a marker. Conventional markers include biocidal markers providing antibiotic resistance and those providing resistance to toxins and heavy metal. 15 Also useful is employing an auxotrophic host and providing prototrophy by complementation. In addition to the conventional selection systems just described, the glycolytic genes of the present invention are particularly desirable markers since they can provide for 20 selection, using sugars as selective substrates, in appropriate mutant host strains.

Other genes may also be inserted into the extra-chromosomal element for a variety of purposes. Where integration is desirable in the genome of the host, a 25 homologous sequence for a particular region of the host genome may be included in the extrachromosomal element. Where amplification of one or more sequences is desired, genes known to provide such amplification, such as dihydrofolate reductase genes, which respond to 30 methotrexate stress or metallothionein genes, which respond to heavy metal stress, may be included in the extrachromosomal element, flanked by the DNA regions to be reiterated. Other regulatory signals may also be

included, such as centromeres, autonomously replicating segments, etc.

In order to isolate the promoters of interest, clones can be made of yeast chromosomal DNA by random digestion or mechanical shearing of the yeast genome. The presence of the desired gene is then determined by introducing a homogeneous clone of a yeast fragment into an auxotrophic host for complementation. Desirably, the cloning vehicle may have another gene which allows for an additional basis for selection, so that double selection techniques can be used. The mutants are substantially incapable of growing on limited nutrient medium, so that one can select for the presence of the desired glycolytic gene by the choice of medium. After isolating the yeast fragment having the desired gene, the fragment may be subcloned so as to remove superfluous DNA flanking regions and provide for a fragment which is more easily manipulated. The smaller fragment containing the desired gene, of a size less than about 500 base pairs may then be further cloned, restriction mapped and sequenced, so as to provide a useful source for the desired promoters and insertion of the alien DNA. Also, as indicated, the promoters in themselves may be useful, in acting as a titrator for repressor or activator, where it is desirable to modulate the production of a particular enzyme in the yeast host. The alien DNA may be from any source, either naturally occurring or synthetic, either prokaryotic or eukaryotic. Of particular interest are mammalian genes which express a poly(amino acid), that is, polypeptide or protein which has physiological activity. To varying degrees, poly(amino acids) prepared in yeast may be modified by glycosylation, where the glycosylation may not occur or may occur at different sites from the naturally occurring mammalian polypeptide and/or in different degrees with different saccharides. It is

therefore of great interest to be able to prepare polypeptides which are different from the naturally occurring polypeptide by the degree and manner of glycosylation and in many instances may differ in one or more ways as to the amino acid sequences, where there may be deletions of one or more amino acids or substitutions of one or more amino acids. Mammalian genes may come from a wide variety of mammalian sources, such as domestic animals (e.g. bovine, porcine, ovine and equine) and primates e.g. humans and monkeys.

As exemplary of the use of the subject promoters in preparing an active polypeptide composition, as well as being of particular interest for a variety of purposes, a protease inhibitor is described and made. The protease inhibitor has the same or substantially the same amino acid sequence of human alpha-1-antitrypsin and is capable of inhibiting a number of proteolytic enzymes. The human alpha-1-antitrypsin gene appears to reside within a 9.6 kb EcoRI DNA fragment in the human genome. The mature mRNA appears to have about 1400 nucleotides. One human alpha-1-antitrypsin cDNA has the sequence shown in FIG. 1B. The predominant form of human alpha-1-antitrypsin is shown in FIG 1A. Other naturally-occurring forms (polymorphisms) are known.

The sequencing of chromosomal DNA coding for alpha antitrypsin has been described by Kurachi et al., Proc. Natl. Acad. Sci. U.S.A., 78, 6826-6830 (1981) and by Chandra et al., Biochem. Biophys. Res. Comm., 103, 751-758 (1981), the disclosures of which are incorporated herein by reference. A primate gene for alpha-1-antitrypsin may be obtained by DNA cloning methods described by Chandra et al., ibid. The gene coding for the predominant form of human alpha-1-antitrypsin, isolated from a human cDNA library by using the baboon

sequence as a DNA hybridization probe is shown in FIG. 1A.

The human alpha-1-antitrypsin has a BamHI restriction site which allows the cutting of the gene with the removal of information for a single glutamic acid from the mature protein. Various schemes can be employed for introducing the human alpha-1-antitrypsin gene adjacent the glycolytic promoter to be under the regulation of the promoter. Where the promoter does not have a convenient restriction site near the f-met codon, the glycolytic gene may be cleaved and chewed back to the promoter with Bal31. A linker may then be introduced downstream from the promoter to provide a convenient cohesive end or flush end for joining to the human alpha-1-antitrypsin gene. The linker can also provide one or more codons for amino acids at the N-terminus of the alpha-1-antitrypsin gene, which may be the same or different from the naturally occurring amino acids.

The gene for human alpha-1-antitrypsin may then be inserted into the extrachromosomal element downstream from the glycolytic promoter, where an f-met codon is provided for initiation of expression of the human alpha-1-antitrypsin.

For example, the cDNA coding for alpha-1-antitrypsin (hereinafter "AT") may then be inserted into an expression vector, such as CTEA32 (FIG. 2), which contains the yeast promoter for triose phosphate isomerase (TPI) inserted at the BamHI site of the shuttle plasmid, CV13 [Broach J.R., Strathern J.N., Hicks J.B., Gene, 8:121-133 (1979)]. A synthetic DNA adaptor was ligated into the TPI promoter after the TPI structural sequences were removed by BAL31 digestion from the KpnI restriction site within the TPI coding region. (Alber et al, J. Molec. Applied Genet., 1,

419-434 (1982)). This adaptor contained an ATG codon for translation initiation, followed by the sequence GAGGATCC. The GAG codon specifies a glutamic acid residue, which is the first amino acid of the naturally-occurring human AT. The GGATCC portion of the adaptor is a cutting site for BamHI endonuclease and allows for the splicing of the remainder of human AT DNA sequence into this vector.

The BamHI site of CTEA32 was constructed to be "in frame" with the rest of the AT structural gene, thereby allowing for the expression of the polypeptide when a BamHI fragment from the cloned cDNA is appropriately inserted into CTEA32. The plasmid consisting of CTEA32 plus the AT gene is called CAT1 (FIG. 2).

- 15 This DNA construct containing the gene for human AT located downstream to a yeast triose phosphate isomerase (TPI) promoter fragment was transformed into yeast strains, N501-B and GK100. Transformation into yeast is described by Beggs, Nature, 275, 104-109 (1978).
- 20 Screening of the transformed yeast strains by immunological assays (competition assays and ELISA assays, using antibodies against alpha-1-antitrypsin) confirmed the presence of large amounts of human AT in yeast made from the plasmid CAT1. The "wild-type" yeast strain, N501-1B (described by Kawasaki et al., Biochem. Biophys. Res. Comm., 108, 1107-1112 (1982)), when transformed with CAT1, produced 1.8 mg alpha-1-antitrypsin per gram of soluble protein (or 0.18% alpha-1-antitrypsin), when grown at 30° on a synthetic minimal medium (modified Wickerham's medium) with 6% glucose. A mutant yeast strain, GK100, when transformed with CAT1, produced 10-15 mg alpha-1-antitrypsin per gram soluble protein (or 1-1.5% alpha-1-antitrypsin) under the same growth conditions. Strains N501-1B and GK100 each carry a defective LEU2 gene which allows for the selective
- 35

maintenance on minimal and leucine-less media of CV13 and CV13-derived plasmids (such as CAT1) which each contain a functional LEU2 gene. When grown on minimal media with only CV13 as a control, N501-1B and GK100 produce no detectable AT. Thus, AT may be specifically produced by the CAT1 plasmid.

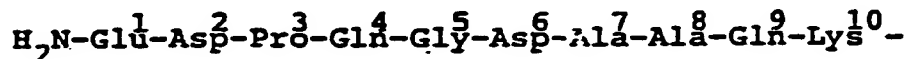
..

Since GK100 produces significantly more AT than N501-1B, it is preferred. However, the present invention is not limited to AT production by GK100. It may be desirable to utilize mutations in GK100 which lead to hyperproduction of AT.

An immuno-adsorption column, made according to the method of Cuatrecasas, P. J. Biol. Chem., 245, 3059 (1970), was prepared by covalently attaching affinity-purified goat antibodies to human AT to CNBr-activated Sepharose. Disrupted GK100 yeast cells were extracted with 3 volumes of phosphate buffered saline pH 7.2 containing 0.5M NaCl, and the extracts were applied to the column. Yeast produced human AT (0.5-1.0 mg) was eluted from the column with 3M NaSCN. After the material was dialyzed to remove salt it was analyzed by electrophoresis on a polyacrylamide gel in the presence of sodium dodecyl sulfate, the results of which are shown in FIG. 3. Based on the relative migration of the protein in the gel, the approximate molecular weight of the human alpha-1-antitrypsin made in yeast is 42,000-43,000 daltons. Naturally occurring human AT has a molecular weight of approximately 54,000 daltons, having a carbohydrate composition of approximately 16% by weight, as shown by Hodges et al, J. Biol. Chem., 254, 8208-8212 (1979). It therefore appears that the yeast produced AT may be unglycosylated or substantially unglycosylated and may lack carbohydrate portions present in the naturally occurring protein.

Alternatively, other expression vectors may be constructed which contain a segment coding for alpha-1-antitrypsin. Such expression vectors may be constructed by methods known to those of ordinary skill in the art using available DNA constructs. A preferred vector is plasmid C1/1, which is more stable than CV13 and CV13 derived vectors, such as CAT1. C1/1 was constructed from plasmid, pJDB248 (Beggs, J., Nature, 275, 104-109 (1978)). The pMB9 sequences were removed from pJDB248 by partial digestion with Eco RI and were replaced by pBR322 DNA which was cut with Eco RI. The restriction map of C1/1 is given in FIG. 4. The C1/1 plasmid contains the entire 2-micron DNA from yeast (S.cerevisiae), with a pBR322 insertion at an EcoRI site. It also contains the LEU2 gene. Thus, the yeast TPI promotor with the adaptor may be inserted into the single BamHI site in the  $Tc^R$  gene of C1/1. Then the AT sequence, attached to a transcription terminator fragment from the yeast TPI gene, may be inserted into the BamHI site downstream from the TPI promotor. The resulting plasmid, HAT4, may then be transformed into N501-1B and GK100 in a manner as described above.

The sequence of the first ten amino acids in the yeast-produced AT may be confirmed by amino acid sequence analysis as identical to the first ten amino acids of the naturally occurring human AT:



The yeast-produced AT does not contain the initiation methionine which is specified by the ATG start codon. Therefore, the yeast cell processes off the methionine to produce the amino acid sequence of natural human AT.

The polypeptides produced according to the present invention having AT activity may be useful for treatment

of a genetic AT deficiency and other diseased states related to inadequate levels of AT. Thus, conditions such as emphysema and other lung disorders related to progressive digestion of lung sacs may be treated, such as, chronic obstructive pulmonary disease or adult respiratory distress syndrome. Non-genetically related emphysema may also be treated, such as, emphysema resulting from heavy smoking. Conditions not necessarily confined to the lungs may also be treated, such as, cystic fibrosis and arthritis. For a review of AT deficiency, see Gadek, J.E., and R. Crystal, "Alpha-1-Antitrypsin Deficiency", The Metabolic Basis of Inherited Disease, J.B. Stanbury, J.B. Wyngaarden, D.S. Fredrickson, McGraw-Hill, N.Y. pp. 1450-67 (1982).

15 The alpha-1-antitrypsin can be used as an antigen for production of polyclonal and monoclonal antibodies to human alpha-1-antitrypsin, for introduction into a host having a deficiency of alpha-1-antitrypsin, or for modulating proteolytic activity in a mammalian host. In 20 Partikular, the alpha-1-antitrypsin can be administered to humans to replace alpha-1-antitrypsin which has been inactivated (oxidized) by tobacco and other smoke.

The polypeptides according to the present invention may be admixed with conventional pharmaceutical carriers. 25 Preferably, the polypeptides are to be administered intravenously or by inhalation. While the effective dosages may vary according to the severity of the condition and weight of the subject, dosages in the range of 0.5-10.0 gm/week of a polypeptide introduced 30 intravenously may, in many cases, be effective. Lower dosages may be effective if the method of administration is by inhalation. Oral administration may also be effective provided the AT is protected in capsules or coated carriers from premature degradation in the 35 digestive tract.



The following examples set forth specific embodiments according to the present invention, but the invention is not intended to be limited thereto.

EXAMPLE 1

5 Strains. Isogenic strains carrying mutations in PGI1, PGK1, GPM1, PYK1, and GCR1 where obtained by ethyl methane sulfonate (EMS) mutagenesis of S. cerevisiae (S. c.) X2180-1A (MATa SUC2 CUP1 gal2, from the Berkeley Yeast Stock Center). 35,000 independent colonies were  
10 grown on YEP-3% glycerol-2% ethanol and were screened by replica plating for the inability to grow on YEP-4% dextrose (Table 1).

Identification of specific lesions was made by complementation tests with known glycolysis mutants (Ciriacy  
15 and Breitenbach, J.Bacteriol, 139:152-60 (1979)), while at least 15 additional complementation groups were found by intercrossing mutant strains. Enzyme assays (Clifton et al. Genetics, 88:1-11 (1980)) confirmed the glycolytic defects in pgi1, pgk1, gpm1, pyk1, and gcr1 mutants.

20 A LEU2 mutant was also derived from S.cerevisiae X2180-1A by EMS treatment and was crossed to X2180-1B (an isogenic MATa strain) to produce N501-1B (MATa leu2 SUC2 CUP1 gal2). Cycloheximide (cyh2) and canavanine (can1) resistances were then selected as spontaneous  
25 mutations in N501-1B. The glycolysis mutants were crossed to N501-1B to produce a series of isogenic leu2 strains each defective in a single glycolytic function or in GCR1.

A tpi1 mutant, S. cerevisiae GLU77 was crossed to  
30 N551-1A (MATa leu2 SUC2 CUP1 gal2); strains derived from this mating were crossed twice to N501-1B to produce a

tpil leu2 strain, N587-2D, which was similar in genetic background to the other Glycolysis mutants.

Mutations in three glucose phosphorylating enzymes produce a strain which is unable to grow on dextrose as the sole carbon source and which is resistant to catabolite repression by 2-deoxyglucose and glucosamine. N517-6C (h<sub>x</sub>k1 h<sub>x</sub>k2 g<sub>l</sub>k1 leu2 can1-100 cyh2 ade2-1) was derived from a h<sub>x</sub>k1 h<sub>x</sub>k2 g<sub>l</sub>k1 strains, D308.3, by screening for glucosamine-resistant spore colonies.

10 Defects in glucose kinasing activities were confirmed by assay.

TABLE I

Complementation Groups of glu<sup>-</sup> Derivatives of X2180-1A

<u>Gene</u>	<u>No. of Mutants</u>	
<u>PYK1</u>	14	
<u>PDC1</u>	9	
<u>GCR1</u>	4	
<u>PGI1</u>	3	
<u>GPM1</u>	3	
<u>PGK1</u>	1	
<u>TPI1</u>	0	
<u>FDP</u>	0	
( <u>LEU2</u> )	(1)	
I	11	
II	10	
III	3	
IV	5	
V	1	
VI	1	
VII	2	
VIII	3	60 other mutations not in the complementation groups
IX	2	
X	3	
XI	2	
XII	1	
XIII	1	
XIV	5	
XV	1	

27 sterile glu<sup>-</sup> strains

35,000 colonies screened (EMS mutagenized for 50% kill)

The homothallic diploid strain, S. c. AB320 was the source of the yeast DNA pool (Nasmyth and Reed, Proc. Nat. Acad. Sci., 77:2119-2123 (1980) and was used as a control in some experiments.

- 5 The triose phosphate isomerase gene (including the upstream sequence having the regulatory signals) is as follows:

[illegible]

The pyruvate kinase gene upstream sequenc having the regulatory signals is as follows:

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10      20      30      40      50      60
GAATTCAGCA TCATAGCTAC GTAAATGTGT TCCGCACCGT CACAAAGTGT TTTCTACTGT
CTTAAGTCGT ACTATCGATG CATTACACAC AGCCGTGCCA GTGTTTCACA AAACATGACA

70      80      90      100     110     120
TCTTTCTTCT TTGGTTTCATT CAGTTCAATT GACTCAGTGC TTTGTTCAAT GCATCTTACC
ACAAACAACA AAGCAAGTAA GTCAATTCAA CTCACTCAGC AAACAAGTTA CCTACAATCG

130     140     150     160     170     180
TAAATGTCAT ATTTTTCCTC TTGGTAAATG AATGCTTGTG ATGTCTTCCA AGTCATTTCG
ATTTTACGTA TAAAAAAGAG AACCATTTAC TTACCAACAC TACAGAAGGT TCACTAAAGC

190     200     210     220     230     240
TTTCCTTCCC ATATGATGCT AGGTACCTTT AGTGCTTCTC TAAAAAAGAA AAAAGCCTCG
AAAGCAAGGG TATACTACGA TCCATGGAAG TCACAGAAAG ATTTTTTTTT TTTTCCGAAC

250     260     270     280     290     300
CCATCAAAAC GATATTCGTT GCGTTTTTTT TCTCAATTAT AAATACTCTT TGCTAACTTT
GGTAGTTTTG CTATAAGCAA CCGAAAAAAA ACACTTAATA TTTATGAGAA ACCATTGAAA

310     320     330     340     350     360
TCATTTCCAA GAACCTCTTT TTTCCAGTTA TATCATGGTC CCCTTTCAAA GTTATTCTCT
AGTAAAGGTT CTTCGAGAAA AAAGGTCAAT ATAGTACCAG GCGAAAGTTT CAATAAGACA

370     380     390     400     410     420
ACTCTTTTTC ATATTCATTG TTTTTCATCC TTTGGTTTTT TATTCTTAAC TTGTTTATTA
TGAGAAAGAG TATAAGTAAG AAAAAGTACC AAACCAAAAA ATAACAATTG AACAAATAAT

430     440     450     460     470     480
TTCTCTGTTG TTTCTATTTA CAAGACACCA ATCAAAACAA ATAAACATC ATCACAATGT
AAGACAGAAG AAGAGATAAT GTTCTGTGGT TAGTTTTGTT TATTTTGTAG TAGTGTTACA

490     500     510     520     530     540
CTAGATTAGA AACATTGACC TCATTAAACG TTGTTGCTGG TTCTGACTTG AGAAGAACCT
GATCTAATCT TTCTAACTCG AGTAATTTCG AACAAAGCAG AAGACTGAAC TCTTCTTGGG

550     560     570     580     590     600
CCATCATTGG TACCATCGGT TCAAGACACCA ACAACCCAGA AACCTTGGTT GCTTTGAGAA
GGTAGTAACC ATGCTAGCCA AGTTTCTGGT TGTTCGGTCT TTGGAACCAA CGAAACTCTT

610     620     630     640     650     660
AGGCTGCTTT GAACATTGTT CGTATGAAGT TCTCTCAGCG TTCTTACCAA TACCACAAGT
TCCGACCAAA CTTGTAACAA GCATACTTCA AGAGAGTGGC AAGAATGCTT ATGCTGTTCA

670     680     690     700
CTGTCTTTGA CAAGCCGACA AAGTCCGAAG AATTGTACCC
GACAGCAACT GTTCCGGTCT TTCAGGCTTC TTAACATGGG

```

Screening of clone bank. The leu2 glycolysis mutants were transformed with a yeast DNA pool inserted into pYE13, a high copy plasmid carrying a selectable LEU2 wild-type gene (Broach et al., Gene, 8:121-133 (1979)).

5 The glycolytic genes were obtained by complementation, involving the simultaneous selection for growth on glucose and leucine prototrophy. A synthetic medium containing yeast nitrogen base, 4% glucose, and the following supplements was used: per liter, 40mg

10 adenine, 20mg arginine, 50mg aspartate, 10mg histidine, 60mg isoleucine, 40mg lysine, 10mg methionine, 60mg phenylalanine, 50mg threonine, 40mg tryptophan, 50mg tyrosine, 20mg uracil, and 60mg valine.

The transformants were purified on leucineless media and

15 were then grown on a non-selective medium (YEPGE) to allow mitotic segregation of the plasmids. Strains which cosegregated the leu2 and glycolysis mutant phenotypes, as determined by replica plating on selective media, were assayed for glycolytic enzyme

20 activities. Yeast DNA preps were made, and the E. coli strain, RR1, was transformed, selecting for ampicillin resistance, to verify the presence of plasmid DNAs in these yeast glycolytic transformants.

Enzyme Assays. The transformed yeast strains were

25 selectively grown on minimal medium with 8% glucose (adenine was added to a final concentration of 50mg/l). The wild-type control, N501-1B, was grown on the same medium plus leucine (100mg/l). The glycolysis mutant strains were grown on YEP-5% glycerol-1% lactate.

30 Overnight cultures were fed fresh media and were aerobically grown at 30° for four hours before harvesting. The cells were washed two times with water and resuspended in 50mM  $K_2HPO_4$  2mM EDTA 3mM 2-mercapto-

35 obtained by vortexing the cells with an equal volume of

glass beads (.45 mm diam.) at high speed for two minutes. The cell debris was removed by centrifugation in a microfuge for 15 min. at 4°. Enzymes were assayed as described by Clifton and Breitenbach, supra. Protein concentrations were determined by the Biuret-TCA method.

#### EXAMPLE 2

In order to determine the activity of the various glycolytic genes in the transformants, the various enzymes were assayed and the results for the 10 transformants were compared to mutant and wild-type strains. The gcr1 mutant had 5-10% of the wild-type levels of most glycolytic activities (exemplified by PGI, aldolase and enolase) and grows very poorly on glucose media. In contrast, the GCR1 transformants had 15 nearly wild-type levels of enzymes and were virtually identical to wild-type for growth on glucose media. The other glycolysis mutants had less than 5% of the normal levels of their respective enzyme activities. However, when transformed with a complementing high copy plasmid, 20 the specific enzyme activities were substantially elevated above wild-type levels (typically 5-10 fold higher). The following Table 2 indicates the results.

TABLE 2

Comparison of Glycolytic Activities in Wild-type, Mutant, and Transformed Strains

<u>Enzyme</u>	<u>Activities</u>			Ratio: .. Transf/Wt
	<u>Wild-type<sup>a</sup></u>	<u>Mutant<sup>b</sup></u>	<u>Transformant<sup>c</sup></u>	
PGI	2.85	.0065	31.49 (10)	11.1
TPI	18.3	.0000	167.8 (10)	9.2
PGK	1.99	.0046	17.67 (3)	8.9
GPM	0.74	.0000	4.80 (10)	6.5
PYK	4.02	.0057	14.77 (10)	3.7

	<u>Wild-type<sup>a</sup></u>	<u>gcr1 Mutant<sup>d</sup></u>	<u>GCR1 Transf<sup>c</sup></u>	
PGI	2.85	.2436	2.42 (10)	.85
Aldolase	4.33	.4415	2.96 (10)	.68
Enolase	0.43	.0274	.316 (10)	.74

<sup>a</sup>Wild-type is N501-1B.

<sup>b</sup>The respective mutant strains are N543-9D (pgi1 leu2), N587-2D (tpi1 leu2), N548-8A (pkgl leu2), N583-2C (gpm1 leu2), and N549-3A (pyk1 leu2).

<sup>c</sup>The activities of the transformants are averages for many different isolates. The numbers in parentheses represent the numbers of independent transformants assayed.

<sup>d</sup>The gcr1 leu2 mutant strain is N525-2C.

EXAMPLE 3

In order to demonstrate that the hyperproduction of glycolytic enzymes was specific to the mutational defect complemented by the particular plasmid, assays for ten



different glycolytic proteins were conducted on the various transformants. The following Table 3 reports the results for one transformant for each of the six different glycolysis genes which were examined in detail.

TABLE 3

RELATIVE ENZYME ACTIVITIES OF WILD-TYPE AND TRANSFORMED

GLYCOLYTIC ENZYMES

<u>Strains</u>	<u>GLK</u>	<u>PGI</u>	<u>PFK</u>	<u>FBA</u>	<u>TPI</u>	<u>GLD</u>	<u>PG</u>
N501-1B	1.00	1.00	1.00	1.00	1.00	1.00	1.0
Transformant GCR-8	1.05	0.63	1.44	0.79	0.62	0.63	0.7
Transformant PGI-19	0.64	5.63	1.26	0.57	0.58	0.75	0.5
Transformant TPI-10	0.99	0.77	1.35	0.99	13.85	0.87	0.6
Transformant PGK-2	0.54	0.45	1.05	0.54	0.46	0.63	2.9
Transformant GPM-2	0.97	0.82	1.69	1.02	1.02	0.85	0.9
Transformant PYK-1	1.02	0.83	1.09	0.89	1.22	0.84	1.2

The GCR-8 transformant gave nearly wild-type levels of all ten enzymes, while PGI-19, TPI-10, PGK-2, GPM-2 and PYK-1 transformants overproduced their respective glycolytic proteins, but not other enzymes.

- 5 It was noted that the plasmids readily segregated (typically 5-50% segregation in fully grown cultures even under selective pressure of leucine prototrophy, so the assayed cultures probably contain cells with a range of number of plasmids. By complementation in E. coli and/or sequencing, TPI1 and PYK1 have both been shown to be the structural gene.

#### EXAMPLE 4

- Exploitation of the promoter for TPI1 for the production of human alpha-1-antitrypsin was demonstrated as follows. The plasmid CV13 was employed. CV13 can be maintained by selection of yeast with an average of about ten copies per cell. CV13 is comprised of pBR322, the replicon for the 2μ-plasmid and the yeast LEU2 gene.
- 20 TPI1 promoter fragment was obtained by cutting the TPI1 gene at the unique KpnI site (bases 511 to 518); and the resulting linearized DNA was then treated with Bal31 for four to five minutes in order to remove the TPI1 structural sequences. Linkers, either EcoRI, Hind III or BamHI, were then inserted. The linkers will then cleave with the appropriate Restriction enzyme to provide cohesive ends for insertion of human alpha-1-antitrypsin genes. The human alpha-1-antitrypsin gene was digested with BamHI, which cleaves at the 5'-terminus of the coding strand to remove the information for a single glutamic acid codon from the mature protein. Four different constructions were prepared, as set forth in the following Table 4. From this table it is noted that the glutamic acid codon is substituted by the codons for alanine and proline in three of the constructions having the initiator methionine.

After ligation of the human alpha-1-antitrypsin construction into the CV13 plasmid, the resulting plasmid was transformed into S. c. N501-1B. The resulting yeast cells were then grown on a minimal synthetic medium.

5

TABLE 4

<u>Plasmid</u>	<u>N-terminal amino acid</u>	<u>Orientation in CV13</u>
CAT1	<u>met glu</u> + hAT*	clockwise
C-Ta2	<u>met ala pro</u> + hAT	counterclockwise
C-Ta1	<u>met ala pro</u> + hAT	clockwise
10 C-TSα2	<u>met ala pro</u> + hAT, but missing part of TPI promoter	counterclockwise

\*remainder of approximately 400 amino acids of human alpha-1-antitrypsin

15 Yeast cells containing the human alpha-1-antitrypsin genes were broken open by vortexing with glass beads (0.45mm) at high speed for 2-3 minutes. The extraction buffer contained 50mM K<sub>2</sub>HPO<sub>4</sub>, 2mM EDTA, 2mM 2-mercaptoethanol and 1mM PMSF (pH7.4) cell debris was removed by  
20 centrifugation and the extracts contain 3-4mg/ml protein as determined by Lowry assays.

The presence of human alpha-1-antitrypsin was determined using a RIA, employing tritium-labeled human alpha-1-antitrypsin and antibody directed against the protein.  
25 The following Table 5 indicates the results.

TABLE 5

## Competition assay for alpha-1 antitrypsin

<u>Plasmid</u>	<u>Tritium Counts</u>	<u>Average Count</u>	<u><math>\alpha</math>-1-AT [<math>\mu</math>g]</u>	<u>Total Protein (<math>\mu</math>g)</u>	<u>%Total Protein</u>
CAT1	46010 52257	49133.5	0.75	420	.18
C-T $\alpha$ 2	12268 13330	12799	3.35	380	.88
C+T $\alpha$ 1	41635 39071	40353	0.95	360	.26
C-TS $\alpha$ 2	66490 70038	68264	0	345	0

<u>Controls**</u>	<u>Counts</u>
0 $\mu$ g $\alpha$ -1	68440
0.25 $\mu$ g $\alpha$ -1	65333
0.5 $\mu$ g $\alpha$ -1	58928
1.0 $\mu$ g $\alpha$ -1	38468
2.0 $\mu$ g $\alpha$ -1	19559
3.0 $\mu$ g $\alpha$ -1	14432
4.0 $\mu$ g $\alpha$ -1	11155
5.0 $\mu$ g $\alpha$ -1	9615

\*Plasmids were grown in yeast strain, N501-1B. 100 $\mu$ l of extracts were assayed.

\*\*Non-radioactive alpha-1-antitrypsin mixed with 100 $\mu$ l of yeast extract (330 $\mu$ g protein)

It is evident from the above results that an immunologically active product is obtained, which is capable of competing with naturally occurring human alpha-1-antitrypsin for antibodies to the native protein.

Furthermore, the expression of the alpha-1-antitrypsin gene is regulated by the TPI promoter, for as is seen, where a portion of the TPI promoter is removed, no alpha-1-antitrypsin is produced. In addition, the  
5 production of the mammalian protein human alpha-1-antitrypsin has not been optimized in the above study, so that the results indicate a minimum production of product which can be further enhanced. Thus, the TPI promoter is found to be an effective promoter for  
10 efficiently producing high yields of expression products of alien DNA.

#### EXAMPLE 5

##### Purification Of Alpha-1-Antitrypsin From Yeast GK100 Yeast Extracts

15 An immuno adsorption column was prepared by covalently attaching affinity-purified antibodies to human alpha-1-antitrypsin to CNBr-activated Sepharose according to the method of Cuatrecasas, J. Biol. Chem., 245, 3059 (1970). Disrupted GK100 cells were extracted with three volumes  
20 of phosphate buffered saline pH 7.2 containing 0.5M NaCl and applied to the column. The column was eluted with 3M NaSCN and the recovered material was analyzed by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate. The results of the electro-  
25 phoresis are shown in FIG. 3. Track 1 contained a mixture of molecular weight standards: a) phosphorylase B, 97,000 daltons; b) bovine serum albumen (BSA), 65,000 daltons; c) ovalbumin, 43,500 daltons; d) carbonic anhydrase, 30,000 daltons; e) soybean trypsin inhibitor,  
30 20,000 daltons; and f) alpha-lactalbumin, 14,000 daltons. Track 3 contains yeast produced AT purified by immuno-adsorption, molecular weight about 42,000 daltons. Track 7 is a sample of naturally occurring AT purchased from Sigma Chemical Company, heavily contaminated by

blood proteins. A major component of Track 7 is human alpha-1-antitrypsin, molecular weight 54,000 daltons.

#### EXAMPLE 6

##### 5      Activity Of Yeast Produced Alpha-1-Antitrypsin          Against Serine Protease Trypsin

As a control, 10 microliters (1 microgram) of a solution of 100 microgram/ml trypsin, 100 microgram (100 microliters) of bovine serum albumin and 100 microliters of 0.05 molar TRIS, pH 8.0 buffer containing 1mM benzoyl-  
10      arginoyl-p-nitroanilide were mixed, and the increase in absorbance at 405 nm was measured over time in a spectrophotometer. The absorbance value of this solution was used as a standard for 100% trypsin activity. Three additional samples were run in duplicate, each contain-  
15      ing 1 µl trypsin and, respectively containing 25 µl AT solution plus 175 µl buffer, 100 µl alpha-1-antitrypsin plus 100 µl buffer, and 200 µl alpha-1-antitrypsin. All samples contain equal concentrations of substrate and bovine serum albumin. The results demonstrated that  
20      utilizing 25 microliters of AT, 73% of the trypsin activity remained, with 100 microliters of AT, 41% of trypsin activity remained and with 200 microliters of alpha-1-antitrypsin, 26% of the trypsin activity remained. This demonstrated that by increasing the levels  
25      of the yeast made AT the trypsin inhibitory activity also increased.

#### EXAMPLE 7

##### Production of Alpha-1-Antitrypsin From Yeast Plasmids With Increased Genetic Stability

30      Increased levels of AT may be obtained by utilizing C1/1, a plasmid which is more genetically stable than

CV13. The C1/1 plasmid contains the entire 2-micron DNA from S. cerevisiae and, therefore, can promote its own replication and maintenance in yeast in the absence of selection for a genetic marker. Also, C1/1 plasmid has  
5 a single BamHI site located in the Tc<sup>R</sup> gene. Transformants carrying C1/1 may be selected in E. coli by ampicillin- or tetracycline-resistance and in yeast by leucine prototrophy. C1/1 contains pBR322 inserted into an EcoRI site of 2-micron DNA and carries the LEU2 gene  
10 described by J. Beggs, Nature, 275, 104-109 (1978).

The yeast TPI promoter (from CTEA32) with the synthetic DNA adaptor (described above) was inserted as a Bgl II - BamHI fragment (about 900 base pairs) into the BamHI site of C1/1. This insertion created a single BamHI  
15 site into which the human AT gene could be spliced for expression in yeast. As in the CAT1 plasmid, when the AT gene (FIG. 1A) is inserted, the resultant plasmid would have an ATG initiation codon followed by a GAG (glutamic acid codon) to allow the production of mature  
20 human AT protein sequence in yeast.

About 700 base pairs of the 3' flanking region of the yeast TPI gene was added after the human AT sequence to assist in transcription termination. The "termination" fragments are sequences from the XbaI to EcoRI sites in  
25 the plasmid TPIC10 (T. Alber and G. Kawasaki, J. Molec. Applied Genet., 1, 419-434 (1982)).

The yeast termination sequences were attached to the human AT gene by using the vector, pUC13, which has multiple cloning sites into which the terminator and AT  
30 DNA's can be separately inserted. The pUC13 plasmid was constructed as described in Vieira, J., and Messing, J., Gene, 19, 259-268 (1982) for vectors, pUC8 and pUC9. The pUC13 plasmid contained the multiple restriction



site, depicted in FIG. 5, at the start of the lac Z gene. To connect the human AT gene to the TPI transcription terminator, the AT cDNA clone (FIG. 1) was inserted as a Pst I fragment into pUC13 at the single  
5 Pst I site. The AT gene was followed by an Xba I site and Eco RI site in the multiple cloning sequence. Between these Xba I and Eco RI sites of pUC13 was inserted the yeast TPI terminator as a 700 base pair Xba I-Eco RI fragment from pTPIC10. The resulting plasmid,  
10 pUCol+FG1, contained a human AT gene with a yeast transcription terminator (See FIG. 6). An Eco RI-Bam HI synthetic DNA adaptor was then added to the Eco RI site of the plasmid, in order to create a Bam HI site on the 5' end of the yeast terminator. By using this adaptor,  
15 the human AT-yeast terminator sequence could be removed by cutting with Bam HI to liberate a fragment of approximately 2100 base pairs. This BamHI fragment was inserted into the C1/1 plasmid containing the TPI promoter with BamHI adaptor. The resulting plasmid,  
20 HAT4, has the TPI promoter, ATGGAGGATCC adapter, human AT gene (from the BamHI site), and TPI terminator inserted into C1/1. The topology of HAT4 is depicted in FIG. 7.

HAT4 was transformed into N501-1B and GK100. On minimal  
25 media with 6% glucose, 2-3% of the yeast soluble protein was alpha-1-antitrypsin at a cell density of nearly 3g per liter (wet weight). Because HAT4 contained C1/1, this plasmid was maintainable in a variety of rich media, including YEPD (1% yeast extract, 2% peptone, and  
30 2% glucose). On rich media 2-3% AT was still produced but at a higher cell density of 10-20g per liter (wet weight). The HAT4 plasmid was maintained without selection in N501-1B for over 30 divisions on rich media with greater than 70% of the cells containing the

plasmid. In GK100 better than 95% of the cells had HAT4 after 30 divisions on rich media. The advantages of using HAT4 over CAT1 were 1) greater plasmid stability, 2) higher levels of AT as a percentage of total protein, 5 3) much greater yields of cells per liter as a result of using rich media, and 4) cheaper costs of rich media compared to synthetic (leucine-less) media. The mutant yeast strain GK100 has been placed on deposit in the American Type Culture Collection, Rockville, Maryland, 10 ATCC No. 20669.

It is evident from the above results that yeast promoters can be efficiently used for the production of foreign proteins by regulating the expression of alien DNA in yeast. The promoters are found to be strong 15 promoters, so as to provide for a high degree of expression. Furthermore, it would appear that the messengers are sufficiently stable as to allow for a significant degree of translation into the desired expression product. Furthermore, by employing the glycolytic 20 promoters and appropriate nutrient media, the expression of the alien DNA can be modulated. In this way, production of the alien DNA can be turned on and off. Thus, the subject invention provides a method for using yeast as efficient host in the production of foreign proteins, 25 where the production may be modulated. In addition, by using the glycolytic regulation gene, one can turn on and off a plurality of glycolytic promoters.

CLAIMS:

1. An extrachromosomal element capable of replication in yeast and containing a yeast promoter capable of regulating the transcription of a glycolytic protein, 5 which protein is triose phosphate isomerase, pyruvate kinase, .. phosphoglucose isomerase, phosphoglycerate mutase, hexokinase 1, hexokinase 2, glucokinase, phosphofructose kinase, and aldolase, or glycolytic regulation protein, said yeast promoter being followed 10 downstream by other than a gene expressing the protein normally regulated by such promoter.
2. An extrachromosomal element according to Claim 1, having a marker for selection in a yeast host.
3. An extrachromosomal element according to Claim 1, 15 having a gene expressing a foreign protein downstream from said promoter and under the regulation of said promoter.
4. An extrachromosomal element according to Claim 1, wherein said promoter is followed by a gene expressing 20 protease inhibitor having substantially the same structure as alpha-1-antitrypsin.
5. An extrachromosomal element according to any of Claims 1, 2, 3 or 4, wherein said promoter is the triose phosphate isomerase promoter or the pyruvate kinase promoter.
- 25 6. An extrachromosomal element according to Claim 5, wherein a gene expressing an enzyme in a metabolic path way of said yeast is present.

7. A yeast cell containing an extrachromosomal element according to any of Claims 1, 2, 3 or 4.
- 8.. A DNA construct comprising a triose phosphate isomerase promoter followed by a gene expressing a foreign protein under the regulation of said promoter.
- 9 . A DNA construct according to claim 8 , wherein said foreign protein is a protein having protease inhibition activity.
- 10 10. A DNA construct according to Claim 9 , wherein the major sequence of said protein is the same sequence amino acids as human alpha-1-antitrypsin.
11. A DNA construct comprising a pyruvate kinase promoter followed by a gene expressing a foreign protein under regulation of said protein.
- 15 12. A method for preparing any foreign protein which comprises: introducing into a yeast host an extra-chromosomal element according to Claims 1, 2, 3, or 4; and growing said yeast host in an appropriate medium and isolating the protein expressed by said foreign DNA.
- 20 13. A yeast cell containing at least a portion of an extrachromosomal element according to any of Claims 1, 2, 3 or 4 integrated into the genome of said yeast cell, wherein said portion includes at least said promoter and said gene.
- 25 14. A yeast cell according to Claim 13, wherein said promoter is the triose phosphate isomerase promoter

15. A protease inhibitor produced by culturing a yeast cell having an extrachromosomal element according to Claim 4.
16. A protease inhibitor produced by culturing a yeast  
5 cell containing at least a portion of an extrachromosomal element integrated into said yeast cell genome, wherein said portion includes a yeast promoter capable of regulating the transcription of a glycolytic protein, which protein is triose phosphate isomerase, pyruvate  
10 kinase, phosphoglucose isomerase, phosphoglycerate mutase, hexokinase 1, hexokinase 2, glucokinase, phosphofructose kinase, and aldolase, or glycolytic regulation protein, and a gene under the transcription regulation of said promoter and expressing a protease  
15 inhibitor having substantially the same polypeptide structure as alpha-1-antitrypsin.
17. A method of producing a polypeptide having the protease inhibition activity of mammalian alpha-1-antitrypsin, comprising the step of growing a culture of  
20 microorganisms transformed by a DNA transfer vector, said vector comprising a segment coding for mammalian alpha-1-antitrypsin.
18. A method according to Claim 17 wherein said culture comprises fungal microorganisms.
- 25 19. A method according to Claim 18 wherein said microorganisms comprise yeast cells.
20. A method according to Claim 19 wherein said yeast cells comprise cells of the mutant strain GK-100.
21. A method according to Claim 19 wherein said yeast  
30 cells contain allelic mutations to GK-100 mutations whereby hyperproduction of said polypeptide is attained.

22. A method according to Claim 19 wherein said vector comprises 2-micron plasmid DNA, plasmid CATI or plasmid HAT4.
- 5 23. A method according to Claim 17 further comprising the steps of extracting said polypeptide from said culture and purifying said polypeptide.
- 10 24. A method according to Claim 23 further comprising the step of altering a suitable vector to include said segment coding for mammalian alpha-1-antitrypsin to form said DNA transfer vector.
- 15 25. A method according to Claim 23 further comprising the step of introducing said DNA transfer vector into yeast.
- 20 26. A method according to any of Claims 20 to 30 wherein said polypeptide comprises the amino acid sequence of naturally-occurring mammalian alpha-1-antitrypsin.
- 25 27. A method according to Claim 31 wherein said polypeptide is the predominant human form of alpha-1-antitrypsin.
28. A substantially unglycosylated polypeptide characterized by the amino acid sequence of mammalian alpha-1-antitrypsin.
- 30 29. Substantially pure, substantially unglycosylated mammalian alpha-1-antitrypsin.
30. A substantially unglycosylated polypeptide characterized by the amino acid sequence of mammalian alpha-1-antitrypsin for use in the treatment of diseased states.

FIG. 1A

0103409

5' GGGGGGGGGGGGGG CA CCA CCA CTG ACC  
 10 20

-24 -20 -10  
 Met Pro Ser Ser Val Ser Trp Gly Ile Leu Leu Leu Ala Gly Leu  
 TGG GAC AGT GAA TCG ACA ATG CCG TCT TCT GTC TCG TGG GGC ATC CTC CTG CCA GGC CTG  
 30 40 50 60 70 80 90

-1 +1 10  
 Cys Cys Leu Val Pro Val Ser Leu Ala Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp  
 TGC TGC CTG GTC CCT GTC TCC CTG GCT GAG GAT CCC CAG GGA GAT GCT GCC CAG AAG ACA GAT  
 100 110 120 130 140 150

20 30  
 Thr Ser His His Asp Gln Asp His Pro Thr Phe Asn Lys Ile Thr Pro Asn Leu Ala Glu Phe  
 ACA TCC CAC CAT GAT CAG GAT CAC CCA ACC TTC AAC AAG ATC ACC CCC AAC CTG GCT GAT TTC  
 160 170 180 190 200 210

40 50  
 Ala Phe Ser Leu Tyr Arg Gln Leu Ala His Gln Ser Asn Ser Thr Asn Ile Phe Phe Ser Pro  
 GCC TTC AGC CTA TAC CGC CAG CTG GCA CAC CAG TCC AAC AGC ACC AAT ATC TTC TTC TCC CCA  
 220 230 240 250 260 270 280

60 70  
 Val Ser Ile Ala Thr Ala Phe Ala Met Leu Ser Leu Gly Thr Lys Ala Asp Thr His Asp Glu  
 GTG AGC ATC GCT ACA GCC TTT GCA ATG CTC TCC CTG GGG ACC AAG GCT GAC ACT CAC GAT GAA  
 290 300 310 320 330 340

80 90  
 Ile Leu Glu Gly Leu Asn Phe Asn Leu Thr Glu Ile Pro Glu Ala Gln Ile His Glu Gly Phe  
 ATC CTG CAG GGC CTG AAT TTC AAC CTC ACG GAG ATT CCG GAG GCT CAG ATC CAT GAA GGC TTC  
 350 360 370 380 390 400

100 110  
 Gln Glu Leu Leu Arg Thr Leu Asn Gln Pro Asp Ser Gln Leu Gln Leu Thr Thr Gly Asn Gly  
 CAG GAA CTC CTC CGT ACC CTC AAC CAG CCA GAC AGC CAG CTC CAG CTG ACC ACC GGC AAT GGC  
 410 420 430 440 450 460 470

120 130  
 Leu Phe Leu Ser Glu Gly Leu Lys Leu Val Asp Lys Phe Leu Glu Asp Val Lys Lys Leu Tyr  
 CTG TTC CTC AGC GAG GGC CTG AAG CTA GTG GAT AAG TTT TTG GAG GAT GTT AAA AAG TTG TAC  
 480 490 500 510 520 530

140 150  
 His Ser Glu Ala Phe Thr Val Asn Phe Gly Asp Thr Glu Glu Ala Lys Lys Gln Ile Asn Asp  
 CAC TCA GAA GCC TTC ACT GTC AAC TTC GGG GAC ACC GAA GAG GCC AAG AAA CAG ATC AAC GAT  
 540 550 560 570 580 590

160 170 180  
 Tyr Val Glu Lys Gly Thr Gln Gly Lys Ile Val Asp Leu Val Lys Glu Leu Asp Arg Asp Thr  
 TAC CTG GAG AAG GGT ACT CAA CGG AAA ATT GTG GAT TTG GTC AAG GAG CTT GAC AGA CAC ACA  
 600 610 620 630 640 650

190 200  
 Val Phe Ala Leu Val Asn Tyr Ile Phe Phe Lys Gly Lys Trp Glu Arg Pro Phe Glu Val Lys  
 GTT TTT GCT CTG GTG AAT TAC ATC TTC TTT AAA GGC AAA TGG GAG AGA CCC TTT GAA GTC AAG  
 660 670 680 690 700 710 720

210 220  
 Asp Thr Glu Glu Glu Asp Phe His Val Asp Gln Val Thr Thr Val Lys Val Pro Met Met Lys  
 GAC ACC GAG GAA GAG GAC TTC CAC GTG CAC CAG GTG ACC ACC GTG AAG GTG CCT ATG ATG AAG  
 730 740 750 760 770 780

230 240  
 Arg Leu Gly Met Phe Asn Ile Gln His Cys Lys Lys Leu Ser Ser Trp Val Leu Leu Met Lys  
 CGT TTA GGC ATG TTT AAC ATC CAG CAC TGT AAG AAG CTG TCC AGC TGG GTG CTG CTG ATG AAA  
 790 800 810 820 830 840





3/10

5' CCCCCCCCCCCCCCAGTGAATCGACA

[illegible]

C10340.

FIG. 1B - page 2

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Ala	Phe	Thr	Val	Asn	Phe	Gly	Asp	Thr	Glu	Glu	Ala	Lys	Lys	Gln	150
GCC	TTC	ACT	GTC	AAC	TTC	GGG	GAC	ACC	GAA	GAG	CCC	AAG	AAA	CAG	
500				510			520			530				540	
Ile	Asn	Asp	Tyr	Val	Glu	Lys	Gly	Thr	Gln	Gly	Lys	Ile	Val	Asp	160
ATC	AAC	GAT	TAC	GTG	CAG	AAG	GGT	ACT	CAA	CGC	AAA	ATT	CTG	GAT	
		550				560		570				580			170
Leu	Val	Lys	Glu	Leu	Asp	Arg	Asp	Thr	Val	Phe	Ala	Leu	Val	Asn	180
TTG	GTC	AAC	CAC	CTT	GAC	AGA	GAC	ACA	GTT	TTT	CCT	CTC	CTG	AAT	
590				600			610			620				630	
Tyr	Ile	Phe	Phe	Lys	Gly	Lys	Trp	Glu	Arg	Pro	Phe	Glu	Val	Lys	190
TAC	ATC	TTC	TTT	AAA	GGC	AAA	TGG	GAC	ACA	CCC	TTT	GAA	CTC	AAG	
		640				650			660			670			200
Asp	Thr	Glu	Glu	Glu	Asp	Phe	His	Val	Asp	Gln	Val	Thr	Thr	Val	210
GAC	ACC	GAG	GAA	GAG	GAC	TTC	CAC	CTG	GAC	CAG	GTG	ACC	ACC	GTG	
680				690			700			710				720	
Lys	Val	Pro	Met	Met	Lys	Arg	Leu	Gly	Met	Phe	Asn	Ile	Gln	His	220
AAG	CTC	CCT	ATG	ATG	AAG	CCT	TTA	CCC	ATC	TTT	AAC	ATC	CAG	CAT	
		730				740			750			760			230
Cys	Lys	Lys	Leu	Ser	Ser	Trp	Val	Leu	Leu	Met	Lys	Tyr	Leu	Gly	240
TGT	AAG	AAG	CTG	TCC	ACC	TCC	GTG	CTG	CTG	ATC	AAA	TAC	CTG	GGC	
770				780			790			800				810	
Asn	Ala	Thr	Ala	Ile	Phe	Phe	Leu	Pro	Asp	Glu	Gly	Lys	Leu	Gln	250
AAT	GCC	ACC	GCC	ATC	TTC	TTC	CTG	CCT	GAT	GAG	GGG	AAA	CTA	CAC	
		820				830			840			850			260
His	Leu	Glu	Asn	Glu	Leu	Thr	His	Asp	Ile	Ile	Thr	Lys	Phe	Leu	270
CAC	CTG	GAA	AAT	GAA	CTC	ACC	CAC	GAT	ATC	ATC	ACC	AAG	TTC	CTC	
860				870			880			890				900	
Glu	Asn	Glu	Asp	Arg	Arg	Ser	Ala	Ser	Leu	His	Leu	Pro	Lys	Leu	280
GAA	AAT	GAA	CAC	AGA	AGC	TCT	CCC	AGC	TTA	CAT	TTA	CCC	AAA	CTG	
		910				920			930			940			290
Ser	Ile	Thr	Gly	Thr	Tyr	Asp	Leu	Lys	Ser	Val	Leu	Gly	Gln	Leu	300
TCC	ATT	ACT	GGA	ACC	TAT	GAT	GTG	AAG	AGC	CTC	CTA	CGT	CAA	CTG	
950				960			970			980				990	
Gly	Ile	Thr	Lys	Val	Phe	Ser	Asn	Gly	Ala	Asp	Leu	Ser	Gly	Val	310
GGG	ATC	ACT	AAG	GTC	TTC	ACC	AAT	GCC	GCT	GAC	CIC	TCC	CGG	GTC	
		1000				1010			1020			1030			320

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Thr	Glu	Glu	Ala	Pro	Leu	Lys	Leu	Ser	Lys	Ala	Val	His	Lys	Ala	330
ACA	GAG	GAC	CCA	CCC	CTG	AAG	GTC	TCC	AAC	CCC	CTG	CAT	AAG	CCT	
1040				1050			1060			1070				1080	
			340												
Val	Leu	Thr	Ile	Asp	Glu	Lys	Gly	Thr	Glu	Ala	Ala	Gly	Ala	Met	
GTG	CTG	ACC	ATC	GAC	GAG	AAA	GGG	ACT	GAA	GCT	GCT	GGG	CCC	ATG	
1090					1100			1110				1120			
							360								
Phe	Leu	Glu	Ala	Ile	Pro	Met	Ser	Ile	Arg	Pro	Glu	Val	Lys	Phe	
TTT	TTA	GAG	GCC	ATA	CCC	ATC	TCT	ATC	CGC	CCC	CAG	GTC	AAG	TTC	
1130			1140				1150			1160				1170	
			370											380	
Asn	Lys	Pro	Phe	Val	Phe	Leu	Met	Ile	Glu	Gln	Asn	Thr	Lys	Ser	
AAC	AAA	CCC	TTT	GTC	TTT	TTA	ATG	ATT	GAA	CAA	AAT	ACC	AAG	TCT	
		1180			1190			1200				1220			
							390					394			
Pro	Leu	Phe	Met	Gly	Lys	Val	Val	Asn	Pro	Thr	Gln	Lys	STOP		
CCC	CTC	TTC	ATG	GGA	AAA	GTG	GTG	AAT	CCC	ACC	CAA				

CTGCCTCTCGCTCCTCAACCCCCCCCCC<sub>3</sub>,

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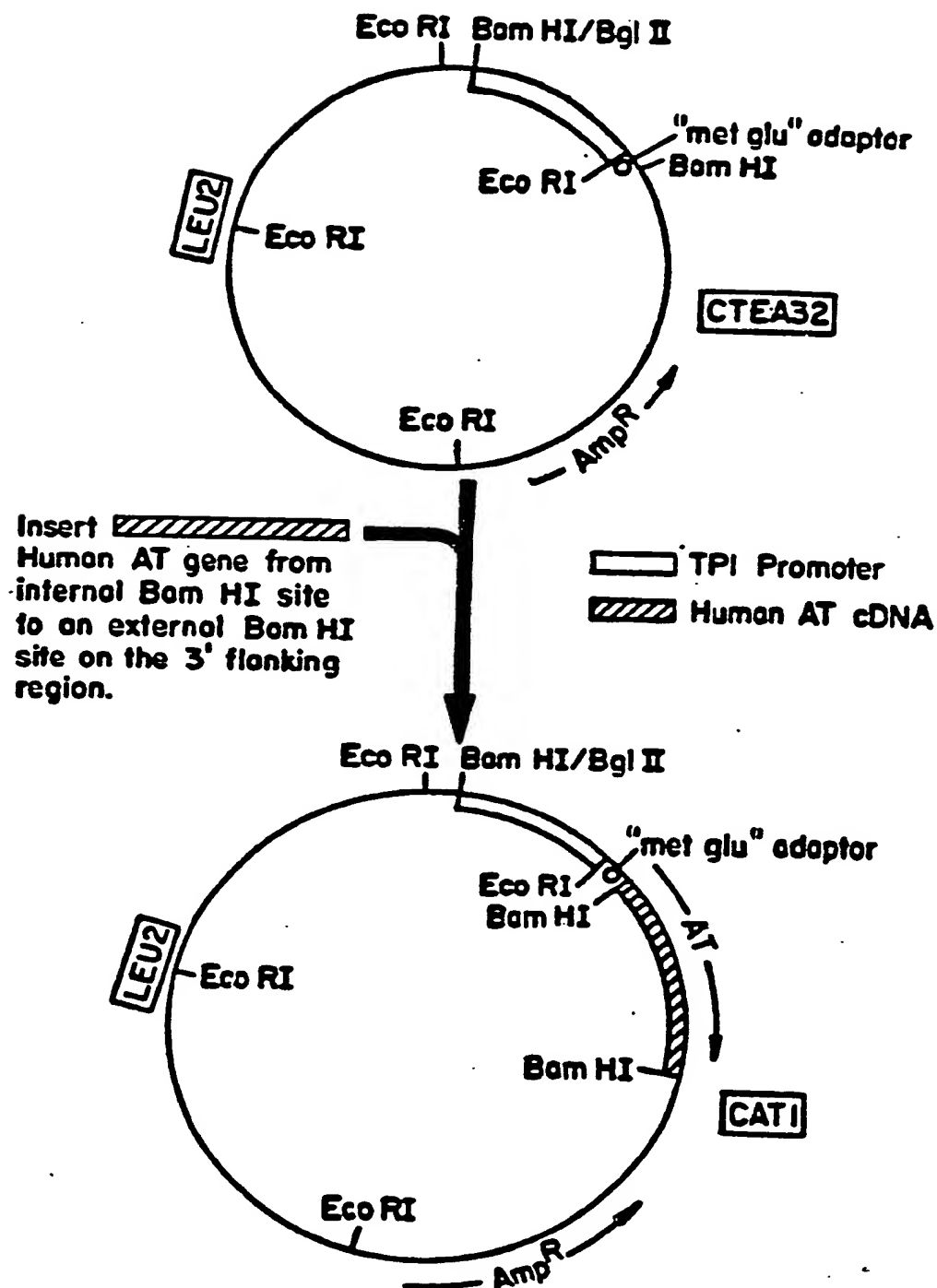


FIG. 2.

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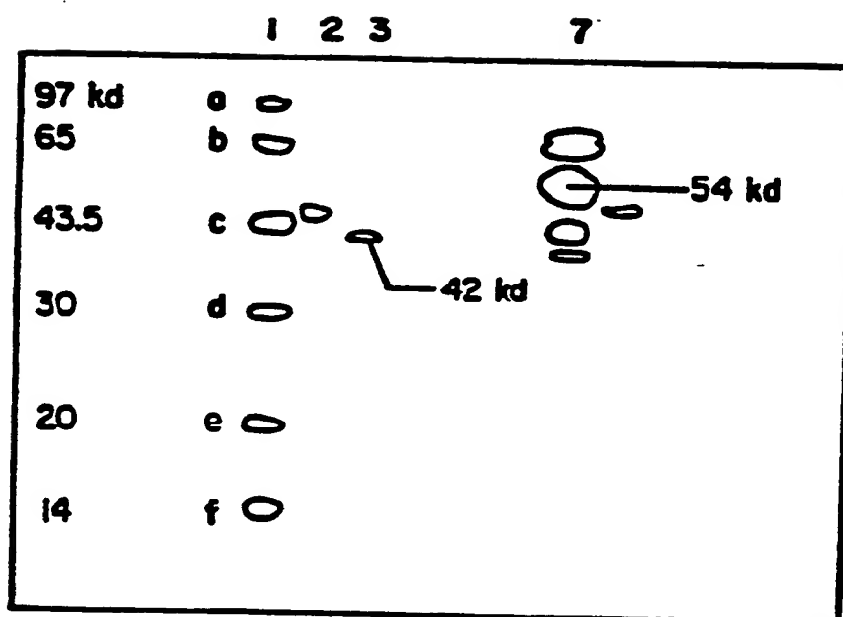


FIG. 3.

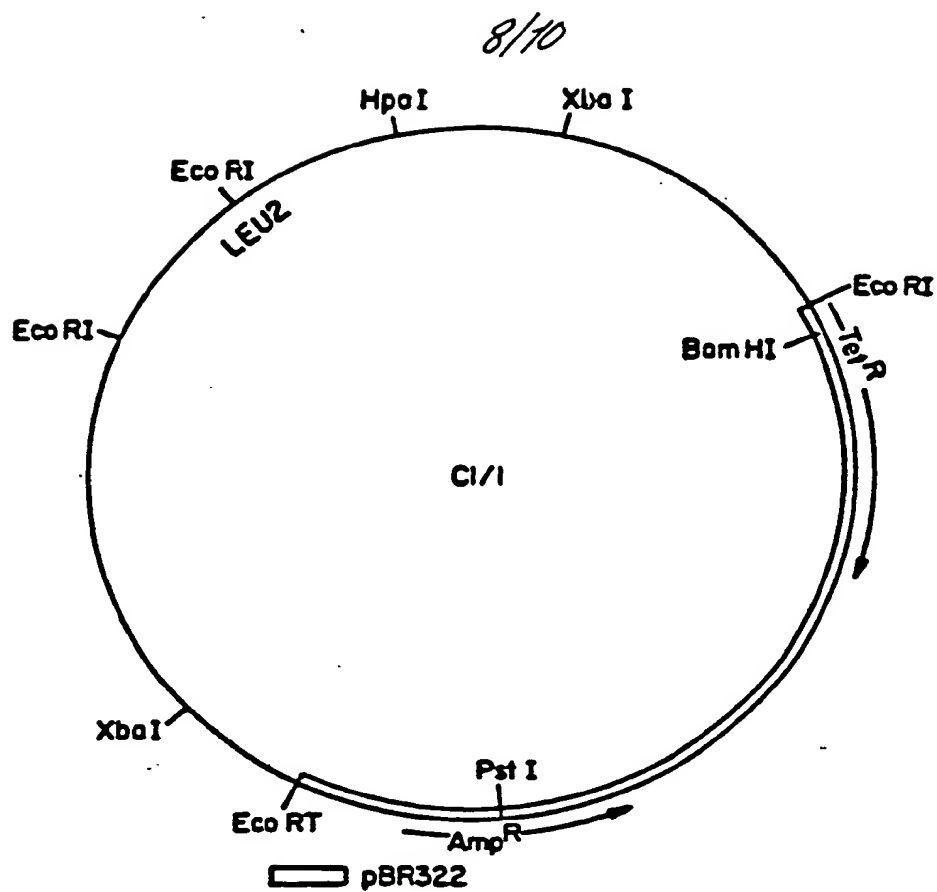


FIG. 4.

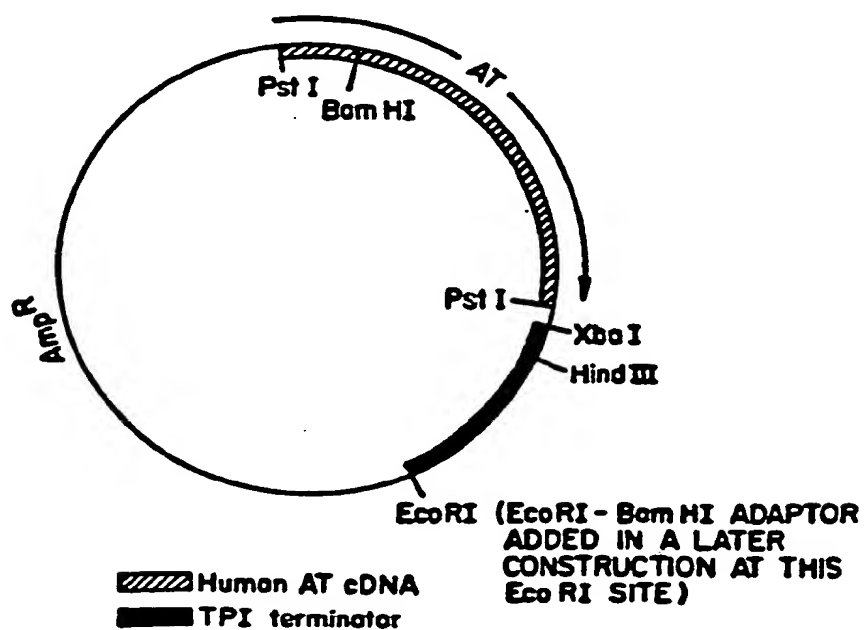


FIG. 6.

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FIG. 5.

M13mp11/pUC13  
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16  
 THR MET ILE THR PRO SER LEU GLY CYS ARG SER THR LEU GLU ASP PRO ARG ALA SER SER 5 6 7 8  
 ATG ACC ATG ATT ACG CCA AGC TTG GGC TGC AGG TCG ACT CTA GAG GAT CCC CGG AGC TCG AAT TCA CTG GCC  
 HindIII PstI Sali XbaI BamHI XmaI SmaI  
 ACCI, HincII  
 EcoRI  
 HaeIII

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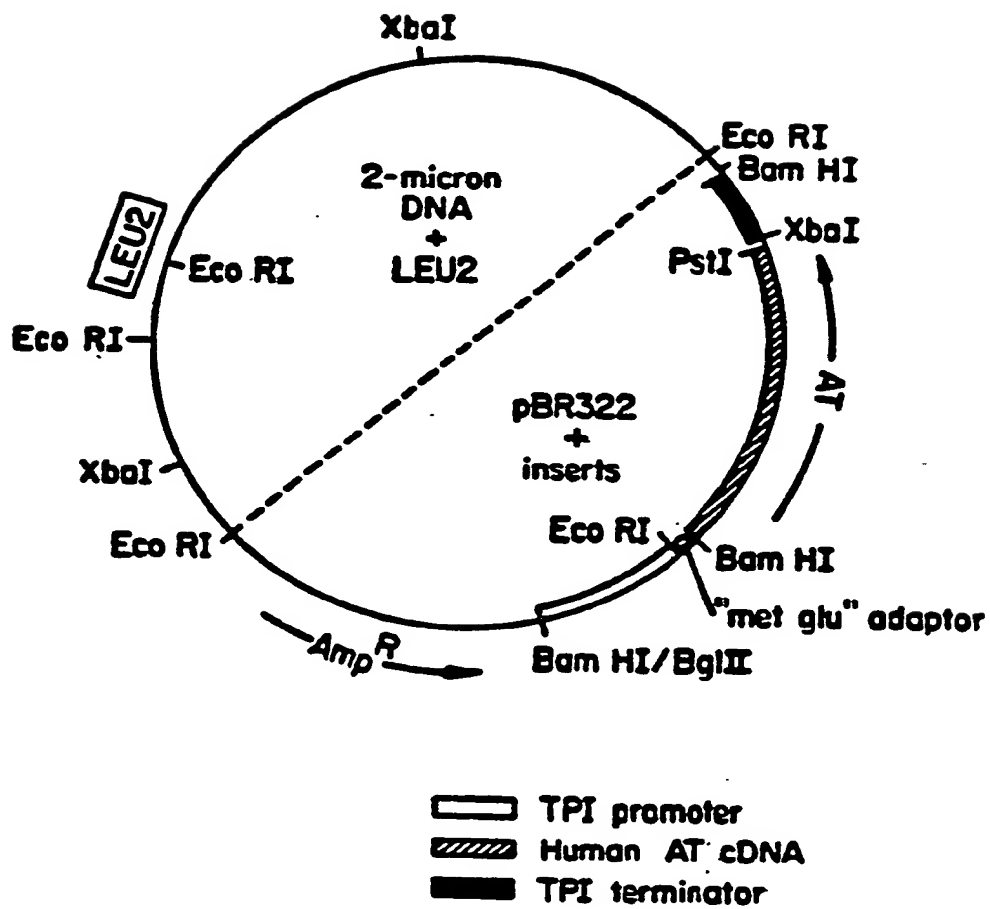


FIG. 7.